FEBS 28375 FEBS Letters 566 (2004) 115–120

Regulation of phosphatidylcholine biosynthesis under salt stress involves choline kinases in *Arabidopsis thaliana*

Guergana Tasseva*, Luc Richard, Alain Zachowski

Physiologie Cellulaire et Moléculaire des Plantes, UMR CNRS 7632, Université Pierre et Marie Curie, 4, place Jussieu, 75252 Paris Cedex 05, France

Received 23 February 2004; revised 8 April 2004; accepted 8 April 2004

Available online 22 April 2004

Edited by Ulf-Ingo Flügge

Abstract **Increasing evidence suggests a major role for phosphatidylcholine (PC) in plant stress adaptation. The present work investigated the regulation of choline, PC and interconnected phosphatidylethanolamine biosynthesis in *Arabidopsis thaliana* L. as a function of cold- and salt- or mannitol-mediated hyperosmotic stresses. While PC synthesis is accelerated in both salt- and cold-treated plants, the choline kinase (CK) and phosphocholine cytidylyltransferase genes are oppositely regulated with respect to these abiotic treatments. Salt stress also stimulates CK activity in vitro. A possible regulatory role of CK in stimulating PC biosynthesis rate in salt-stressed plants is discussed.**

Keywords: Abiotic stress; Phosphatidylcholine synthesis; Differential gene expression; Choline kinase; *Arabidopsis thaliana*

1. Introduction

Phosphatidylcholine (PC) is the major phospholipid in eukaryotic cell membranes, and it has major structural and functional roles. As a substrate for membrane-bound desaturases in the endoplasmic reticulum, PC actively contributes to the formation of the pool of free polyunsaturated fatty acids (see [1] for a review). In plants, PC is a precursor for the synthesis of glycerolipids, such as monogalactosyldiacylglycerol, digalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol, in plastid membranes [2]. Moreover, PC also serves as a reservoir for lipid second messengers, e.g. lyso-PC, phosphatidic acid, diacylglycerol, lyso-phosphatidic acid, in higher eukaryotes [3,4].

There are two PC biosynthesis pathways in higher eukaryotes, the cytidyldiphosphate-choline (CDP-Cho) pathway and the methylation pathway (Fig. 1). The CDP-Cho pathway includes three steps, the first one catalyzing the phosphorylation of Cho, while the methylation pathway requires free, phospho- or phosphoryl-conjugated ethanolamine (EA) moieties to produce PC. The first pathway is the primary route for PC synthesis in mammals [5], while the second operates in a limited mammalian cell types, such as hepatocytes, adipocytes or pituitary cells. In the yeast *Saccharomyces cerevisiae*, both pathways exist, although PC is synthesized primarily by the methylation pathway (see [4] for a review). In plants, it is generally accepted that PC is produced through a mixed CDP-Cho and methylation pathway [6]. The biosynthesis of Cho (Fig. 1) results from the methylation of EA moieties, which can occur at the free base, the phospho-base or the phosphatidyl-base levels depending on plant species or tissue (see [7] for a review).

PC is thought to be involved in adaptive response to abiotic stresses in higher plants. Freezing tolerance is correlated with the amount and the degree of polyunsaturation of PC in planta [8,9] and high salt stress induces a rapid increase in the PC turnover in suspension-cultured cells [10]. From these data it seems that the pool of PC might be tightly regulated and is critical to maintain cell structure and function under stress.

Drought, high salinity and cold stresses are the primary causes of crop loss worldwide [11]. Some plants have evolved specific mechanisms to protect against osmotic stress by accumulating Cho-derived osmoprotective products such as glycine betaine (GB) [7,12]. Although related to Cho metabolism, the adaptive accumulation of GB is not accompanied by a change in the net PC biosynthesis rate [13]. Much effort has been undertaken to improve stress resistance in model species, such as tobacco and *Arabidopsis*, that do not possess the Cho monooxygenase nor the betaine aldehyde dehydrogenase enzymes necessary for GB biosynthesis (see [14] for a review). In Cho monooxygenase transgenic tobacco, significant accumulation of GB occurs only after exogenous Cho supply or by engineering plants to overproduce Cho, and it appears that Cho biosynthesis is a major limiting factor in GB synthesis [13,15–17]. Therefore, genetic engineering revealed differences between natural plant accumulators and non-accumulators of GB with regard to their respective capacities to regulate the Cho and the connected PC biosynthesis fluxes.

Because PC seems to function in adaptive mechanisms in species that do not accumulate GB, we chose to study the metabolism of PC in planta to identify regulatory targets that

* Corresponding author. Fax: +33-1-44-27-61-51.
E-mail addresses: guergana.tasseva@snv.jussieu.fr (G. Tasseva), luc.richard@snv.jussieu.fr (L. Richard), alain.zachowski@snv.jussieu.fr (A. Zachowski).

Abbreviations: AAPT, aminoalcoholphosphotransferase; Cho, choline; CK, choline kinase; CCT, CTP-phosphocholine cytidylyltransferase; CDP, cytidyldiphosphate; CTP, cytidyltriphosphate; EA, ethanolamine; ECT, CTP-phosphoethanolamine cytidylyltransferase; EK, ethanolamine kinase; GB, glycine betaine; NMT, *N*-methyltransferase; PC, phosphatidylcholine; PCho, phosphocholine; PE, phosphatidylethanolamine; PEA, phosphoethanolamine; PEAMT, phosphoethanolamine *N*-methyltransferase; PLD, phospholipase D

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.04.015

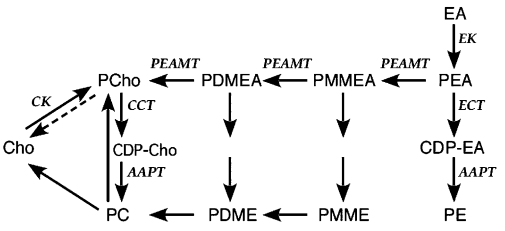

Fig. 1. Simplified scheme of Cho and PC biosynthesis pathways in higher plants. The conversion of phospho-bases (PEA; PMMEA, phosphomonomethylethanolamine; PDMEA, phosphodimethylethanolamine; PCho) to phosphatidyl-bases (PE; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyldimethyletha- nolamine; PC) implicates cytidine 5′-diphosphate (CDP)-containing intermediates, some of which being omitted for simplicity. The CDP-EA pathway for PE biosynthesis is also indicated. Genes related to metabolic steps are indicated in italic. For nomenclature, see abbreviations.

are differentially altered by stress. Here we show that changes in the PC biosynthesis rate in *Arabidopsis thaliana* are correlated to changes in the expression level of PC pathway genes. In vivo and in vitro labeling experiments corroborate some of these gene expression data, and identify the first step in the CDP-Cho pathway as a rate-controlling step for PC biosynthesis in plants exposed to salinity.

2. Materials and methods

2.1. Plant material and stress treatments

Arabidopsis thaliana L. ecotype Columbia seeds were sown on stainless metal filters in close contact with a mixture of soil and sand (5:3, v/v). Plants were grown at 22 °C under continuous illumination (50 μmol/m^2/s) for 4 weeks up to a vegetative pre-bolting phase. At the time of stress treatments, the soil mixture surrounding the roots was gently removed by careful washing and plants were transferred in hydroponic medium containing one half-strength Murashige and Skoog medium (0.5× MS) for 1 h prior to treatment. All further treatments were done under the same light conditions.

For cold treatment, plants were transferred from 22 to 4 °C in pre-chilled 0.5× MS. For hyperosmotic treatments, plants were transferred in 0.5× MS containing iso-osmotic concentrations of 200 mM NaCl (salt stress) or 400 mM mannitol (simulating water stress) at 22 °C. Control plants were maintained in non-supplemented 0.5× MS at 22 °C.

After various time periods, plants were gently blotted dry and whole rosettes were either used for metabolic labeling analysis or rapidly frozen in liquid nitrogen prior to Northern-blot analysis.

2.2. Metabolic labeling experiments

Whole rosettes (0.5 g fresh weight) from control and from 24- or 48-h treated plants were cut in fragments using a razor blade and placed in small vials containing 0.5× MS, supplemented with either 0.5 μCi/ml [*methyl*-^{14}C]Cho chloride (56 mCi/mmol, Amersham Biosciences) or 0.5 μCi/ml [2-^{14}C]EA hydrochloride (55 mCi/mmol, Amersham Biosciences). Rosette fragments were vacuum infiltrated for 5 min at 600 mm Hg and then incubated for 10 min at 22 °C, or 4 °C for cold stress, under gentle agitation. Following infiltration, plant material was gently rinsed with 0.5× MS solution and further incubated in 0.5× MS at 22 °C (or 4 °C for the cold-treated plants) for various periods of tune (0, 20, 60 and 120 min). After incubation, rosette fragments were gently blotted dry and rapidly frozen in liquid nitrogen.

2.3. Analysis of the in vivo-labeled Cho/EA-containing metabolites

Samples were ground in liquid nitrogen and immediately extracted for 2 h with a methanol/chloroform mixture (2:1, v/v) under nitrogen atmosphere at 4 °C. Samples were filtered through Whatman glass fiber filters. An aliquot was removed for the determination of total [^{14}C]Cho or [^{14}C]EA uptake, and subsequently, one volume of a mixture containing chloroform/150 mM NaCl (1:1, v/v) was added to facilitate phase separation. The organic phase was washed once with glacial methanol/water mixture (5:4, v/v) for better phase separation, then evaporated to dryness under a nitrogen stream and dissolved in chloroform. For Cho labeling experiments, the aqueous phase was neutralized, dried in vacuo and the resulting residue was dissolved in a minimal volume of deionized water (pH 5.0). Phospholipids in the organic phase of either [^{14}C]Cho- or [^{14}C]EA-labeled samples were analyzed by thin layer chromatography (TLC) on silica gel 60A plates (Merck) with the solvent mixture containing chloroform, methanol, acetone, acetic acid, water (100:20:40:20:8, v/v), whereas the aqueous metabolites in the Cho labeling experiment were separated by TLC with the solvent mixture containing methanol, 0.5% NaCl, ammonium hydroxide (50:50:1, v/v). Radiolabeled metabolites were visualized using a PhosphorImager scanner (Molecular Dynamics), identified relatively to the migration of commercial standards spotted on the same plates and the label incorporation associated with each compound was determined by scraping appropriate areas prior to scintillation counting.

2.4. In vitro choline kinase (CK) assay

CK activity was assessed in the cytosolic subcellular fraction. One part of fresh weight of rosettes was ground in glass mortar with two parts of an extraction medium containing 50 mM Tris–HCl, pH 7.4, 0.32 M saccharose, 0.3 M $MgCl_2$ and 1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 10 000 × *g* for 10 min and the supernatant (cytosolic fraction) was recovered. After determining protein concentration as described using bovine serum albumin as standard [18], enzyme assays immediately proceeded. The reaction mixture contained 100 mM Tris–HCl, pH 8.5, 8 mM $MgCl_2$, 8 mM ATP, 0.18 mM [^{14}C]Cho chloride (56 mCi/mmol, Amersham Biosciences) in a final volume of 200 μl. The reaction was initiated by the addition of the protein aliquot (5–30 μg/assay). After 5–15 min incubation at 30 °C, the reaction was terminated by boiling samples immediately in a water bath for 5 min. Radiolabeled Cho and phosphocholine (PCho) were analyzed as described above for in vivo labeling experiment.

2.5. RNA isolation and Northern-blot analysis

Pairs of specific forward/reverse primers were used to generate genomic DNA fragments from individual genes by polymerase chain reaction using standard conditions: 5′-TGGAACGCGACTCGTCG-3′/5′-GGGAAGAAGCAGTTAGC-3′ (At1g34100), 5′-CAAACG-CTAGGTTCGAGC-3′/5′-CGTTTGAGTGTATGTCTC-3′ (*AtCK1*), 5′-CTAGATTAGTAAGGATG-3′/5′-CAAAACCTTACGAGAA-G-3′ (At1g74320), 5′-CAAACTTGTACGGTATG-3′/5′-AGTTCTA-GGTCAGAGAG-3′ (At2g26830), 5′-GACTTCTCTCCAACTCT-3′/5′-AACAAGAAGCTTCCTAC-3′ (At4g09760); 5′-CGTAGACAGT-ATCCAAG-3′/5′-GAGAGAGAGTGCGAGGC-3′ (*AtCCT1*), 5′-GATGGGATTCAGCAACG-3′/5′-ATGTGAGCAACAAAGGG-3′ (*AtCCT2*), 5′-AGATAATCCGTATTCTG-3′/5′-TAGTATGTAGCA-TTTGG-3′ (*AtECT*); 5′-GTACTCGTCTCTTGA-TC-3′/5′-CCAACA-AGAAAGTAACC-3′ (*AtAAPT1*), 5′-CAAGTTCGTGTATGTTG-3′/5′-AAGCAACTAGAAGCTTC-3′ (*AtAAPT2*); 5′-GGAACAGAG-GAAGAGAG-3′/5′-GATTTTCAAGTCTGGAG-3′ (At1-g48600), 5′-TCCCTGGTCTTACTCTC-3′/5′-CATATGAGTCATTCTCG-3′ (At1g73600); 5′-TCAACATGATCTCATAG-3′/5′-AGGACTCTAG-TCCTTGG-3′ (*AtNMT1*). Primers for two stress-marker genes were 5′-AGTTCGTATTACAGAGGAGCTCAGGGT-3′/5′-ACGAGCA-GCAGTAGCTCGACGAGGTA-3′ for the At1g43890 (*RAB18*) and 5′-TGATGAGTCTCCTGATCAGAAGCCAGG-3′/5′-GGCACATC-CTTGTCGATATCAGATTCG-3′ for the At5g52310 (*RD29A*).

Total RNAs were isolated from samples using the guanidinium thiocyanate–cesium chloride purification method as described [19]. Samples of 20 μg RNA were fractionated on a 1.2% agarose–formaldehyde gel and blotted on Nytran SuperCharge transfer membrane (Schleicher & Schuell). Probes were prepared using the Megaprime™ DNA Labelling System according to the manufacturer's instructions (Amersham Biosciences). Prehybridization (1 h) and hybridization (16 h) were performed at 65 °C as described [20]. Following hybridization, membranes were washed twice in 2× saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) (w/v) for 10 min at room temperature, then once in 1× SSC, 0.1% SDS (w/v) for 15 min at 65 °C. After soaking, membranes were wrapped in saran wrap and exposed to a phosphor screen overnight. Labeled bands were revealed using a

PhosphorImager scanner and quantified by ImageQuant (Molecular Dynamics).

3. Results and discussion

3.1. Differential effects of abiotic constraints on the rates of PC and phosphatidylethanolamine (PE) biosynthesis in Arabidopsis

The two pathways for PC formation in plants are the CDP-Cho and the methylation pathways. The CDP-Cho pathway requires free Cho, while the methylation pathway requires free EA. This latter pathway is also the main route for Cho synthesis in *Arabidopsis* [21] and in GB-producing plants like spinach [22] (Fig. 1). The fluxes of each pathway were determined in whole rosettes of 4-week-old plants, normally grown or exposed to abiotic constraints, by measuring the incorporation of either radiolabeled Cho or EA into PC (Fig. 2A). The flux of the CDP-EA pathway was determined by measuring the incorporation of labeled EA into PE (Fig. 2B).

Independent of treatment, the in vivo incorporation of [^{14}C]Cho into lyso-PC never exceeded 0.5% of total label recovered in the lipid phase, while incorporation of [^{14}C]EA into PC was at most 2.5%. On the basis of rate incorporations into PC of labeled Cho and EA, respectively, the methylation pathway accounted for approximately 10% of PC synthesis in control plants, and approximately 6%, 1% and 9% in cold-, salt- and mannitol-stressed plants, respectively (data not shown). These data denote a weak contribution of the methylation pathway for PC synthesis in *Arabidopsis* under either condition. Fig. 2 presents the incorporation of labeled Cho and EA into PC and PE, respectively, as the mainly labeled lipid compounds in the corresponding in vivo labeling experiments. After 60 min of in vivo labeling, about 52% and 42% of the labeled Cho were taken up into PC for cold- and salt-treated plants, respectively, against 27% of incorporation observed for control plants (Fig. 2A). In contrast, plants treated with mannitol did not show any increase in the rate of PC biosynthesis relatively to the control (Fig. 2A). On the other hand, the rate of biosynthesis of PE from labeled EA was similar in control and cold-treated plants, while it was reduced up to 4-fold in salt-stressed or in water-stressed plants (Fig. 2B).

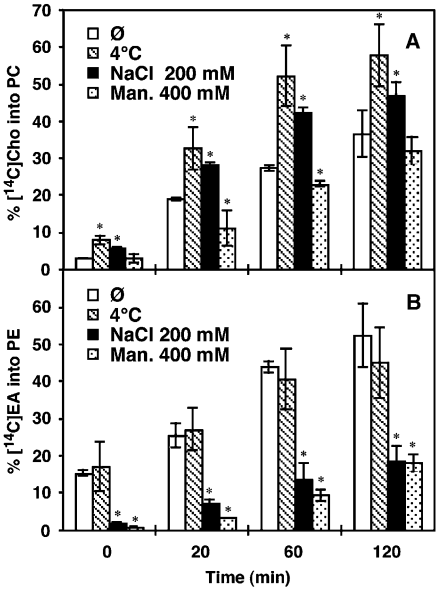

Fig. 2. Effects of various abiotic treatments on the in vivo biosynthesis rate of PC and PE. Four-week-old plants were subjected to cold (4 °C), salt (200 mM NaCl), or mannitol (400 mM Man, simulating water stress) treatment. Rosette fragments from control (Ø) or 24-h treated plants were vacuum infiltrated with either [methyl-^{14}C]Cho chloride or [2-^{14}C]EA hydrochloride. Incorporations of radiolabeled Cho and EA after the indicated incubation time are presented as percent of label recovered in the PC (A) and the PE (B), respectively, over the total label per sample. The latter represents the sum of label incorporated into all classes of aqueous and lipid intermediates. Results are means of three separate experiments ± S.D. Similar lipid labeling pattern was observed for 48-h treated plants. The results were statistically analyzed by Student's *t*-test. Significant differences were accepted if $P < 0.05$ (*).

Our present data reveal different responses of the two interconnected pathways for PC and PE biosynthesis to abiotic treatments. Whereas synthesis of PC via the CDP-Cho pathway is accelerated in response to either cold or saline treatment, synthesis of PE via the CDP-EA pathway is repressed by either hyperosmotic treatment and is not significantly altered by cold. An accelerated biosynthesis rate may contribute to an increase in the content of PC in membranes, as previously reported for cold-stressed *Arabidopsis* plants [23] or, alternatively, to maintain sufficient levels of PC when subjected to enhanced turnover.

The stimulation of PC biosynthesis in vivo may be the consequence of up-regulation of specific steps of the CDP-Cho pathway. We expect that changes in the pool of each of the pathway intermediates should inform us about the nature of such regulatory steps. Therefore, individual analysis of metabolites was undertaken for each treatment (Fig. 3). Comparatively to the control, the reduced pool size of PCho observed under all treatments indicates an acceleration of the cytidyltriphosphate (CTP)-phosphocholine cytidylyltransferase (CCT)-mediated conversion of PCho to CDP-Cho. The step catalyzed by aminoalcoholphosphotransferase (AAPT) was not limiting, since only traces of CDP-Cho were detected at any condition. Our in vivo labeling indicates positive regulation of CCT, which may be at a transcriptional [23,24] or post-transcriptional level [5,25,26]. However, our data cannot

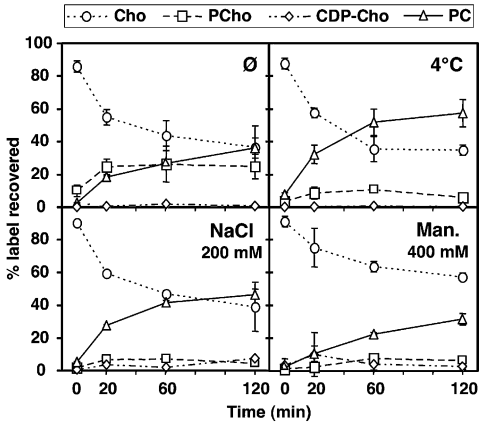

Fig. 3. Effect of cold-, salt- and mannitol-mediated hyperosmotic stresses on the pool sizes of CDP-Cho pathway intermediates. Time course of in vivo [^{14}C]Cho label distribution among water-soluble derivatives (Cho, PCho, CDP-Cho) and PC, in control and 24-h treated plants. Results are means of three separate experiments ± S.D. Similar labeling pattern was observed for 48-h stressed plants.

give insight whether the step catalyzed by CK is also positively affected under these abiotic stresses.

These results suggest that the enhanced synthesis of PC triggered by salt and cold stress is due to an accelerated CDP-Cho pathway, and does not involve the methylation pathway. Therefore, the CDP-Cho pathway must be up-regulated in order to adapt its flux rate to the increased demand for PC formation. Previous studies on the metabolism of PC in cold-treated plants showed that regulation may occur at the transcriptional level [23,27]. We therefore measured transcription of enzymes involved in the PC and the interconnected PE biosynthesis to identify the regulatory mechanisms of hyperosmotic or cold treatment.

3.2. CK genes are up-regulated by hyperosmotic stress

We measured the effect of abiotic stresses on transcript levels of four gene families involved in the PC and the interconnected PE biosynthesis, namely CKs/ethanolamine kinases (EKs), CCTs/CTP-phosphoethanolamine cytidylyltransferases (ECT), AAPTs and phosphoethanolamine *N*-methyltransferases (PEAMT, also called *N*-methyltransferases (NMT)). As positive control, two well-documented stress-marker genes, the abscisic acid-regulated *RAB18* (for responsive to abscisic acid) and the abscisic acid-independent *RD29A* (for responsive to desiccation) [28–31] were used. As shown in Fig. 4C, the *RAB18* mRNA level peaked at 24 h of hyperosmotic treatment, while *RD29A* transcript peaked at 24 h of treatment whatever the applied stress, therefore establishing the reliability of our experimental conditions.

Cold regulation of CCT has been already described [23] and our results confirmed the stimulation *of AtCCT2* expression within 6 h of cold treatment. This was not observed for *AtCCT1* (Fig. 4A). These genes were not induced by hyperosmotic treatments. Although the reaction catalyzed by CCT is considered as the rate-limiting step in the CDP-Cho pathway in animals [5,26] and in plants [9,32–34], the increased rate of PC biosynthesis in salt-treated plants appears to be not related to up-regulation of the *CCT* genes.

We expected that CCT activity would be up-regulated under cold as well as under hyperosmotic stress conditions, as demonstrated by in vivo measurements of pool sizes of PC precursors (Fig. 3). However, the steady-state transcript levels of both *CCT* genes was not altered by hyperosmotic stress, suggesting post-translational regulation. Such a mechanism exists in animals [5,26] and was already reported in pea stems, where CCT activity is rate-limiting for PC synthesis [25,32]. Potential regulatory mechanisms include a change in the subcellular distribution of the enzyme, such as a translocation from the microsomal fraction to the cytosol increasing its activity [25]. Alternatively, allosteric regulation via CMP, could inhibit CCT activity [25]. Additional mechanism include a decrease in protein levels resulting from auxin treatment [25].

The *AtECT* gene is another member of the *CCT* gene family and its transcript level increased slightly following cold treat-

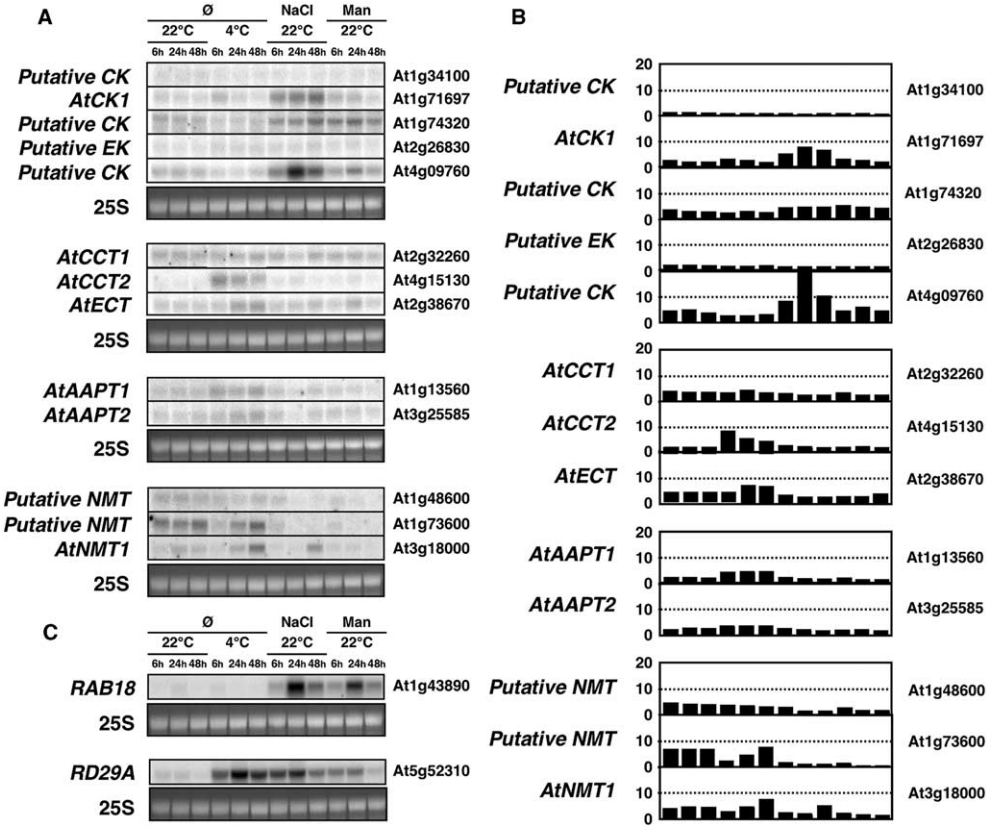

Fig. 4. Changes in the steady-state levels of transcripts related to the PC and the interconnected PE biosynthesis pathways in response to various abiotic constraints. (A) Northern-blot analysis of 4-week-old plants exposed to low temperature (4 °C), 200 mM NaCl (NaCl) or 400 mM mannitol (Man) for the indicated periods of time. 25 S ribosomal RNAs are shown for normalization. (B) Relative steady-state levels of transcripts normalized with respect to the 25S rRNA. (C) Northern-blot analysis of *RAB18* and *RD29A* transcripts reflecting the responsiveness of plants toward the applied abiotic constraints. Data are representative of three independent experiments.

ment but not in response to hyperosmotic treatments. Both *AtAAPT* genes behaved in a similar manner to *AtCCT2*. Among genes associated to the methylation pathway, the putative *NMT* genes, At1g73600 and At3g18000, showed a reduction in their mRNA level up to 3-fold after 6 h of cold treatment, but returned to normal levels at 48 h. The key enzyme in the plants methylation pathway PEAMT contributes to Cho synthesis and to PC formation. It controls the metabolic flux to Cho since *Arabidopsis* mutants deficient in a *PEAMT* gene expression have a severe reduction up to 64-fold in Cho content [21]. Consistently, tobacco plants overexpressing *PEAMT* produce 5-fold more PCho and 50-fold more free Cho, while unaffecting the amount of PC [13]. These findings suggest that PEAMT regulates the Cho synthesis rate but do not affect the pool of PC. Data reported here also denotes that PEAMT activity is not correlated with the PC synthesis rate since salt-treated plants showing an enhanced rate of PC synthesis (Fig. 2A) contain a lower level of *PEAMT* transcripts (Fig. 4A and B).

In addition to *AtCK1*, four genes encode putative CKs and EKs in *Arabidopsis*. Transcripts from each of these genes showed striking variability in their steady-state level under stress treatment. The putative *CK* At1g34100 and the putative *EK* At2g26830 showed equal mRNA levels under all experimental conditions. In contrast, the other three *AtCK* genes were differentially responsive to saline or mannitol treatments. *AtCK1* mRNA level increased 2.5- to 4-fold between 6 and 48 h of salt stress, peaking at 24 h of treatment, but was not responsive to mannitol-mediated hyperosmotic stress. While slightly stimulated under mannitol treatment, the At4g09760 transcript level increased dramatically up to 4.5-fold after 24 h of saline treatment. Interestingly, the expression pattern of this gene was similar to the one *of RAB18* under hyperosmotic treatment, suggesting a possible common relationship in their regulation. The pattern of At1g74320 transcripts in response to hyperosmotic treatment resembles that of At4g09760 as both showed a slight reduction in transcript levels in the cold-treated plants. This suggests a positive regulation of *CKs* that may be related to the enhanced synthesis of PC in response to salinity.

Based on sequence homology, it is likely that the *CK* genes encode kinases with a strict specificity for Cho substrate, suggesting a crucial role of the CDP-Cho pathway versus the methylation pathway in regulating PC biosynthesis during hyperosmotic stress adaptation. However, the existence of CK isoforms in mammals displaying dual specificity for both Cho and EA [35–37] supposes further biochemical investigations before understanding the relative importance of one or another metabolic functions of the *Arabidopsis* CKs in adaptive mechanisms. Several data favor the hypothesis that plants have separate CK and EK activities carried out by distinct proteins. On the one hand, kinase isoforms purified from spinach leaves [38] or soybean seeds [39] exhibit specificity towards EA exclusively and, on the other hand, two CK isoforms in soybean produced in heterologous systems show negligible EA kinase activity [40]. Therefore, it is likely that the regulation of some CK isoforms in response to hyperosmotic stress might specifically modulate PC biosynthesis throughout the CDP-Cho pathway under such conditions without contributing to PE biosynthesis. However, until accurate functional enzymatic analysis of the *CK* gene products is provided, we cannot definitively exclude that these products may catalyze the phosphorylation of EA in *Arabidopsis*.

Biosynthesis of PC in animals is regulated by both CK and CCT (see [41] for a review). Indeed, stimulated CK activity is required for recycling Cho into phospholipase D (PLD)-mediated PC turnover signaling pathways in mammals [42]. In yeast, a change in the phosphorylation state of CK directly affects the pool of PC, demonstrating that post-translational modifications of CK regulates PC synthesis through the CDP-Cho pathway [43]. Since *AtCK1* is inducible by wounding in *Arabidopsis* [44], our results denote that CKs also represent a potential regulatory target for some abiotic constraints in plants, such as increased environmental salinity.

3.3. Increased CK activity in rosettes from salt-stressed Arabidopsis *plants*

In betaine-accumulating plants such as spinach, Cho is diverted through the formation of GB and CK specific activity remains essentially unchanged upon salinization [22]. Therefore, it is interesting to examine the regulation of CK activity in *Arabidopsis*, which does not accumulate GB. In order to check whether transcriptional activation of the *CK* genes is reflected at the enzymatic level, we assayed in vitro CK activity. As shown in Fig. 5, total CK activity was enhanced by salt stress by approximately 1.5- and 2-fold after 24 and 48 h of NaCl treatment, respectively. Other treatments did not induce significant change in CK activity.

The concomitant increase in *CK* mRNA levels (Fig. 4A and B) and CK activity in response to salinity denotes regulation at a transcriptional level that may contribute in the stimulation of the biosynthesis rate of PC in vivo (Fig. 2A). However, control at a transcriptional level is probably not a single mechanism modulating CK activity since, unlike salt stress, mannitol-mediated hyperosmotic stress induced no detectable increase in CK activity in vitro (Fig. 5), nor in the PC biosynthesis rate (Fig. 2A), despite a noticeable increase in the mRNA steady-state levels of two *CK* isogenes (Fig. 4A and B). These *CK* isogenes, At1g74320 and At4g09760, may thus be considered as hyperosmotic stress-responsive, whereas activation of *AtCK1* is specifically related to salt stress. Thus, it is probable that such regulation of the *CKs* depends on signaling pathways that differ according to the ionic or the osmotic nature of the stress (see [45] for a review). The discrepancy between the enhanced transcript levels and the unresponsiveness of the CK enzymes towards mannitol treatment raises the question of a possible phosphorylation-mediated post-translational regula-

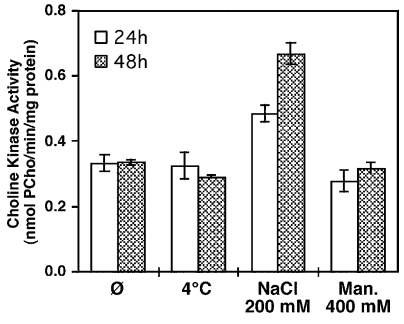

Fig. 5. Effect of abiotic treatments on the in vitro CK activity. Crude cytosolic extracts were prepared from whole rosettes of control plants or plants submitted to abiotic stress for 24 and 48 h and assayed in vitro for CK activity. Values represent the average of three separate experiments ± S.D.

tion of the CKs, as observed in yeast [43], that would selectively affect the CK isoenzymes under hyperosmotic stress.

The inverse responsiveness of the *CK* genes comparatively to the *CCT* genes under salt and cold stresses indicates that PC homeostasis is regulated by two distinct mechanisms. Both mechanisms are supposed to maintain high levels of PC for membrane structure remodeling and/or production of lipid messengers. Salt stress activates PLDs in various plant species (see [46,47] for reviews) and possibly in response to cold in *Arabidopsis* [48]. Furthermore, it is likely that the biosynthesis as the PLD-mediated turnover of PC is accelerated by saline treatment since, despite the existence of an increased CK activity, no change in the pool size of labeled Cho between control and stressed plants was detected (Fig. 3). We therefore hypothesize that PLD-mediated PC turnover is enhanced under salt stress. In such a way, the activation of CKs would be required for recycling the released Cho moieties into the CDP-Cho pathway, as in animals [42]. In contrast, no induction of phospholipase A_2-mediated deacylation of PC was observed in response to either treatment because we failed to detect in vivo-labeled glycerophosphocholine nor a significant change in the labeled lyso-PC pool. An alternative hypothesis would be to consider that high salt concentration versus low temperature distinctively alters the activity of the CKs and the CCTs enzymes. In this respect, CCTs would be sensitive to cold, as confirmed for AtCCTs [23], whereas CKs would be sensitive to salt. Transcriptional up-regulation would be induced to compensate enzyme sensitivity and fulfill requirement of PC under such adverse conditions. Comparative in vitro enzyme assays of purified CKs and CCTs would clarify this.

The present study shows that abiotic stresses trigger differential regulation of the CDP-Cho pathway for PC biosynthesis. Taken together, the biochemical and molecular data denote a regulatory role of the CKs in salt tolerance mechanisms within non-accumulating GB species like *Arabidopsis*. Further characterization of the salt-stress targeted *CK* genes using engineered plants will allow us to define precisely their biological role and foresee possible agronomic application to remedy saline environment.

Acknowledgements: This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS), the Ministère de l'Education Nationale, de la Recherche et des Technologies, and the Université Pierre et Marie Curie (UMR 7632). We greatly thank Dr. David Jackson for editing the English text.

References

[1] Browse, J. and Somerville, C.R. (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 467–506.
[2] Ohlrogge, J. and Browse, J. (1995) Plant Cell 7, 957–970.
[3] Exton, J.H. (1990) J. Biol. Chem. 265, 1–4.
[4] Kent, C. and Carman, G.M. (1999) Trends Biochem. Sci. 24, 146–150.
[5] Vance, D.E. (2002) in: Biochemistry of Lipids, Lipoproteins and Membranes (Vance, D.E. and Vance, J., Eds.) Phospholipid Biosynthesis in Eukaryotes, pp. 205–232, Elsevier, Amsterdam.
[6] Kinney, A.J. (1993) in: Lipid Metabolism in Plants (Moore, T.S., Ed.) Phospholipid Head Groups, pp. 259–284, CRC Press, Boca Raton, FL.
[7] Rhodes, D. and Hanson, A.D. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 357–384.
[8] Sikorska, E. and Kacperska-Palacz, A. (1980) Physiol. Plant. 48, 201–206.
[9] Kinney, A.J., Clarkson, D.T. and Loughman, B.C. (1987) Biochem. J. 242, 755–759.
[10] Pical, C., Westergren, T., Dove, S.K., Larsson, C. and Sommarin, M. (1999) J. Biol. Chem. 274, 38232–38240.
[11] Boyer, J.S. (1982) Science 218, 443–448.
[12] Sakamoto, S. and Murata, N. (2000) J. Exp. Bot. 51, 81–88.
[13] McNeil, S.D., Nuccio, M.L., Ziemak, M.J. and Hanson, A.D. (2001) Proc. Natl. Acad. Sci. USA 98, 10001–10005.
[14] Chen, T.H.H. and Murata, N. (2002) Curr. Opin. Plant Biol. 5, 250–257.
[15] Nuccio, M.L., Russell, B.L., Nolte, K.D., Rathinasabapathi, B., Gage, D.A. and Hanson, A.D. (1998) Plant J. 16, 487–496.
[16] Nuccio, M.L., Rhodes, D., McNeil, S.D. and Hanson, A.D. (1999) Curr. Opin. Plant Biol. 2, 128–134.
[17] Huang, J., Hirji, R., Adam, L., Rozwadowski, K.L., Hammerlindl, J.K., Keller, W.A. and Selvaraj, G. (2000) Plant Physiol. 122, 747–756.
[18] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[19] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[20] Church, G.M. and Gilbert, W. (1984) Proc. Natl. Acad. Sci. USA 81, 1991–1995.
[21] Mou, Z., Wang, X., Fu, Z., Dai, Y., Han, C., Ouyang, J., Bao, F., Hu, Y. and Li, J. (2002) Plant Cell 14, 2031–2043.
[22] Summers, P.S. and Weretilnyk, E.A. (1993) Plant Physiol. 103, 1269–1276.
[23] Inatsugi, R., Nakamura, M. and Nishida, I. (2002) Plant Cell Physiol. 43, 1342–1350.
[24] Jones, P.L., Willey, D.L., Gacesa, P. and Harwood, J.L. (1998) Plant Mol. Biol. 37, 179–185.
[25] Price-Jones, M.J. and Harwood, J.L. (1986) Biochem. J. 240, 837–842.
[26] Clement, J.M. and Kent, C. (1999) Biochem. Biophys. Res. Commun. 257, 643–650.
[27] Qi, Q., Huang, Y.F., Cutler, A.J., Abrams, S.R. and Taylor, D.C. (2003) Planta 217, 547–558.
[28] Mantyla, E., Lang, V. and Palva, E.T. (1995) Plant Physiol. 107, 141–148.
[29] Yamagushi-Shinozaki, K. and Shinozaki, K. (1993) Mol. Gen. Genet. 236, 331–340.
[30] Yamagushi-Shinozaki, K. and Shinozaki, K. (1993) Plant Physiol. 101, 1119–1120.
[31] Yamagushi-Shinozaki, K. and Shinozaki, K. (1994) Plant Cell 6, 251–264.
[32] Price-Jones, M.J. and Harwood, J.L. (1983) Biochem. J. 216, 627–631.
[33] Kinney, A.J. and Moore, T.S. (1987) Arch. Biochem. Biophys. 259, 15–21.
[34] Rolph, C.E. and Goad, L.J. (1991) Physiol. Plant. 83, 605–610.
[35] Ishidate, K., Furusawa, K. and Nakazawa, Y. (1985) Biochim. Biophys. Acta 836, 119–124.
[36] Uchida, T. and Yamashita, S. (1990) Biochim. Biophys. Acta 1043, 281–288.
[37] Aoyama, C., Nakashima, K., Matsui, M. and Ishidate, K. (1998) Biochim. Biophys. Acta 1390, 1–7.
[38] Macher, B.A. and Mudd, J.B. (1976) Arch. Biochem. Biophys. 177, 24–30.
[39] Wharfe, J. and Harwood, J.L. (1979) Biochim. Biophys. Acta 575, 102–111.
[40] Monks, D.E., Goode, J.H. and Dewey, R.E. (1996) Plant Physiol. 110, 1197–1205.
[41] Kent, C. (1995) Annu. Rev. Biochem. 64, 315–343.
[42] Ishidate, K. (1997) Biochim. Biophys. Acta 1348, 70–78.
[43] Yu, Y., Sreenivas, A., Ostrander, D.B. and Carman, G.M. (2002) J. Biol. Chem. 277, 34978–34986.
[44] Titarenko, E., Rojo, E., Leon, J. and Sanchez-Serrano, J.J. (1997) Plant Physiol. 115, 817–826.
[45] Zhu, J.K. (2001) Curr. Opin. Plant Biol. 4, 401–406.
[46] Munnik, T. and Meijer, H.J.G. (2001) FEBS Lett. 498, 172–178.
[47] Wang, X. (2002) Curr. Opin. Plant Biol. 5, 408–414.
[48] Wang, X., Wang, C., Zheng, S.L. and Qin, C. (2000) Biochem. Soc. Trans. 28, 813–816.